#14 6/10/02

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Cole et al.		
Serial No.	:	09/508,710	Examiner:	David H. Kruse
Filed	:	July 10, 2000	Group Art Unit:	1638
For	:	NEW PLANT GENES		

DECLARATION UNDER 37 C.F.R. § 1.131

Ian Cummins declares as follows:

1. I am an inventor of the above-identified patent application.

2. Prior to August 11, 1997, the invention described and claimed in the subject application was completed as evidenced by the following.

3. Prior to August 11, 1997, Ian Cummins isolated RNA encoding the glutathione transferase subunits of the present invention. A copy of the relevant lab notebook pages in which Ian Cummins described this work are attached hereto as Exhibit A. The dates shown on these pages are redacted.

4. Prior to August 11, 1997, Ian Cummins synthesized cDNA library from the isolated RNA described in Exhibit A and purified the cDNA library. A copy of the relevant notebook pages in which Ian Cummins described this work are attached hereto as Exhibit B. The dates of Exhibit B are redacted.

5. Prior to August 11, 1997, Ian Cummins sub-cloned the cDNA library into expression vectors and transformed *E. coli* cells with the expression vectors. A copy of the

relevant notebook pages in which Ian Cummins described this work are attached hereto as Exhibit C. The dates of Exhibit C are redacted.

6. Prior to August 11, 1997, Ian Cummins checked the cDNA library expression vectors for the presence of glutathione transferase subunits by binding to a glutathione resin and by western blot analysis. A copy of the relevant notebook pages in which Ian Cummins described this work are attached hereto as Exhibit D. The dates of Exhibit D are redacted.

7. Prior to August 11, 1997, Ian Cummins showed by western blot analysis that certain cDNA's expressed glutathione transferase subunits. A copy of the relevant notebook pages in which Ian Cummins described this work are attached hereto as Exhibit E. The dates of Exhibit E are redacted.

8. Prior to August 11, 1997, Ian Cummins sequenced the cDNA encoding for glutathione transferase subunits and showed that they were novel DNA sequences encoding novel glutathione transferase subunits. A copy of the relevant notebook pages in which Ian Cummins described this work are attached hereto as Exhibit F. The dates of Exhibit F are redacted.

Ian Cummins

Date: May 30th 2002

IAN Cummins

UNIVERSAL OFFICE SUPPLIES

DUPLICATE BOOK

IAN CUMMINS.
WHOLE CUT
BOOK 3.

BIOLOGICAL SCIENCES,
UNIVERSITY OF DURHAM,
DURHAM,
DH1 3LE,
U.K.

Re-Order Code DUPA4U

Concentrate gel isolated protein + run an aliquot on a gel.
→ ≈ 150 µl. Run 5 µl on gel.

Freeze - diluted over-expressed from 10 + 11 (originally 2 ml of each 2 x 1 ml tubes)

1 tube of each → -20°C for next boost.
1 tube of each → 200 µl IMS. 50 µl use 2 µl + 1 µl on gel.

Also mix with 25 KD isolated protein.

Use 20 µl of 18H with 2 µl of overexpressed protein.

Gel.
1) SDS 7B.
2) Gel isolated protein 5 µl.
3) Marker.
4) 18H 20 µl.
5) 18H 20 µl.
6) 24P 20 µl.
7) 2 µl #11 Overexpressed + 20 µl 18H
8) 1 µl overexpressed. #11
9) 2 µl overexpressed #11
10) 18H 20 µl.

Run @ 150V + silver stain

Blot OK. 24 KD is pure.
Overexpressed band is 26 KD.

Prepare reagents / glassware / plasticware for RNA prep. from stored shoots.

TRIZOL PREP.
10 vols. trizol.
-20

CHLOROFORM ONLY
IPA
75% EtOH

1) Homogenise 10-20 vols. 5 g (50 ml)
2) 5 min @ R.T.
3) Add 0.2 ml $CHCl_3$ / ml TRIZOL (10 ml)
4) Shake 15 sec. RT 2-3 min.

RNA protocol cont. RNA was extracted with P/C/IAA pH 4 x 1, C/IAA x 1 + EtOH pptd.

8) Inc. RT 10 min.
9) 12,000g 10 min. 4°C. → RNA pellet.
10) Wash 75% EtOH (1 ml 2 wash for 1 ml mix)
11) Vortex + cent. 7,500g 5 min 4°C.
12) Air dry resuspend in DEPC H_2O + check it out.

↳ 1 ml, Scan 1/100
A260 = 0.36
A280 = 0.23
= 1.56

A_1 = 40 µg/µl. 1.44 µg/µl
RNA → −80°C. Aliquots.

Poly(A)+ selection.

Buffer to make – 1 x Washing Buffer. 10 mM TRIS-HCl pH 7.5
150 mM LiCl
1 mM EDTA.

75 µg RNA → ≈ 1.5 µg A+.

1) Start with 75 µg RNA in 100 µl.
2) → 65°C 2 min.
3) Use 200 µl Beads + add to Epp. in Magnet. After 30 secs remove SN + wash with 100 µl 2x Binding Buffer. (PINK).
4) Then remove from magnet + add 100 µl 2x Binding Buffer
5) Add RNA + mix – stand 5 min RT.
6) Magnet 30 secs. Remove SN.
7) Wash 2x 200 µl wash buffer.
8) Add elution buffer → 65°C, 2 min → magnet + remove SN to new tube. Can add RNasin + store −80°C.

Wavelength: 280.0

Function	Reading
Scan	0.22848

Scan directory: VI
Start wl: 220.0 nm
End wl: 300.0 nm
Overlay scans: [No

]
]
e: 1
[sec]

Method name: A:\DEFAULT
Autosave name: [A:\]SCANS
Sampling device: None
Read average time: 0.50 sec

A:\WORK_003

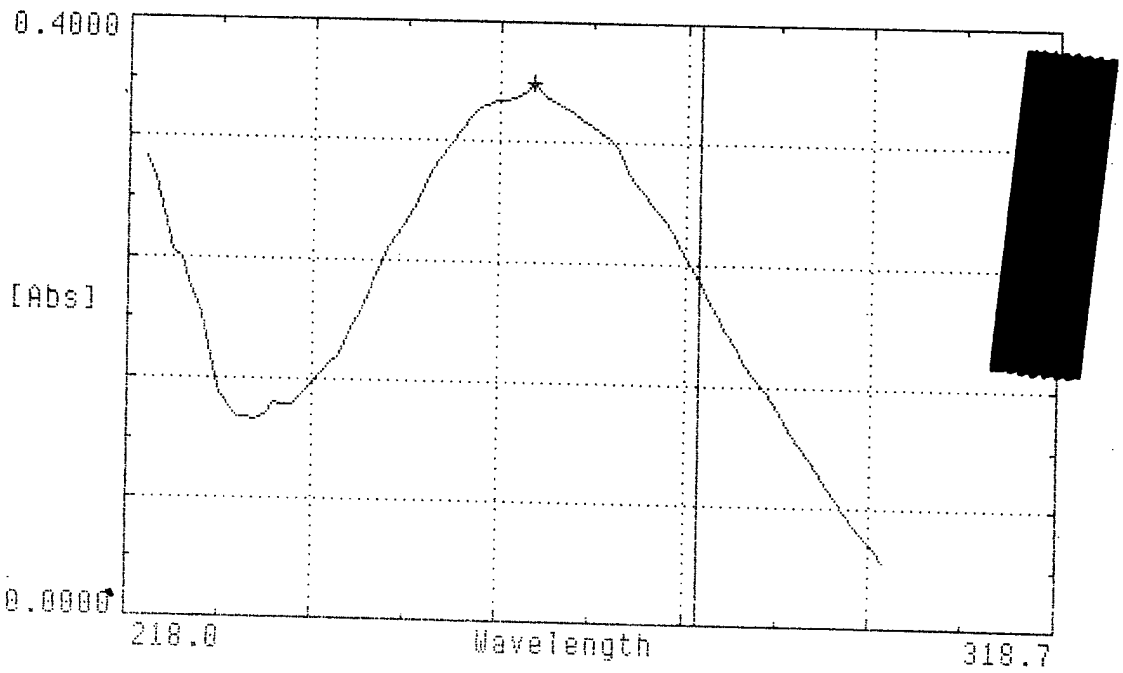
Print ↓ ↑ ← → Exit

A:\WORK_003 Scan

wl	Abs	Pick
262.0	0.3603	pk

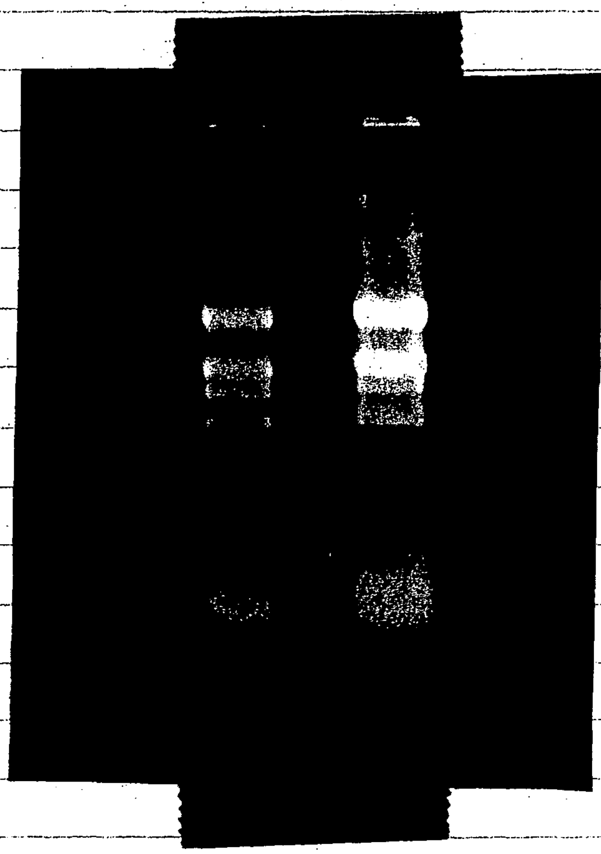
Zoom ZoomOut Trace Function Autoscale Annotate Print

Functions: Scan



Ran 2µl + 5µl RNA + eq. vol of 100% formamide on 1.2% agarose TBE gel, 75V 1hr.

Stain 15min
EtBr 0.5µg/ml.



RNA @ 1.44µg/µl.
for 75µg
52µl + 48µl H2O.
→ 100µl per sample.

try using 100µl each
= 144µg.
→ ≈ 1→2µg.
∴ Do 5 gels
for ≈ 5µg.

Looks OK - some poly(A)+.

Prepare wash solution (1M Tris 7.5 stock, DEPC treated EDTA).

		stock	For 10ml	1ml
1x WASH Buffer —	10mM Tris-HCl pH7.5	1M	100µl	.10
	150mM LiCl	8M	188µl	18.8
	1mM EDTA	0.5M	20µl	2
				969

308µl
9,692µl
10,000µl.

Pooled 100µl RNA x2
130µl RNA (#3) aliquot = 8µl each. (→24µl)
3×8µl aliquots.
→ -80°C.

Also pooled unbound → -80°C.

Wavelength Scan
ReadSamples   Tabulate   +-*Scans   Scatt

Scan directory: VIEW   Autoprint: [No
Start wl: 220.0 nm   Autosave: [No
End wl: 320.0 nm   Scans per samp
Overlay scans: [No]   Interval: 5.00

A:\WORK_001   5μl of 40

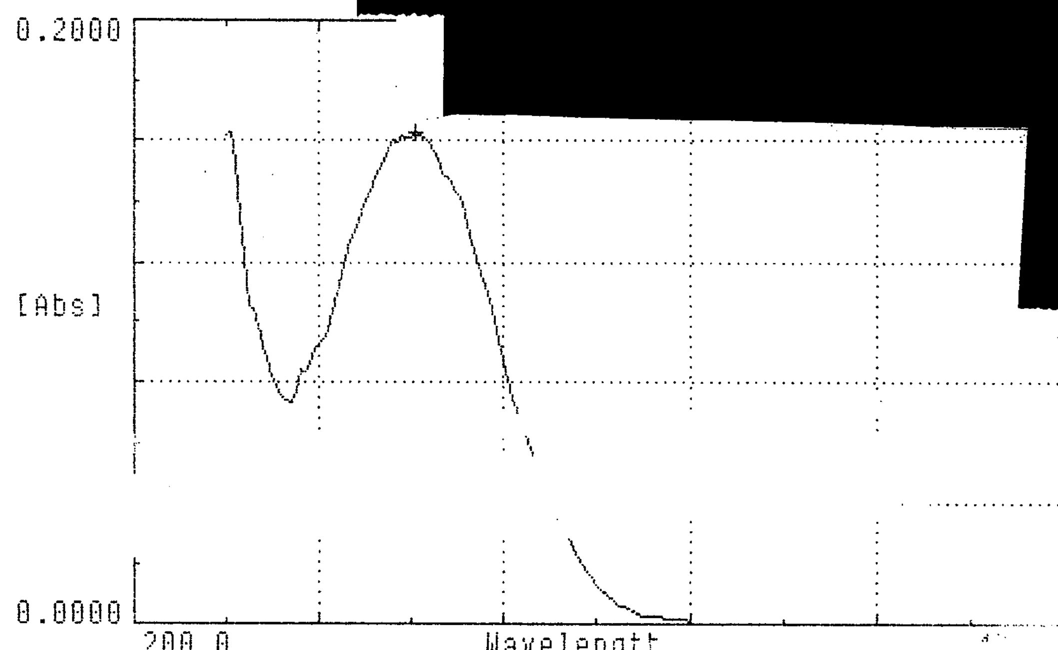
Print ↓ ↑ ← → Exit   Zoom   ZoomOut   Trac

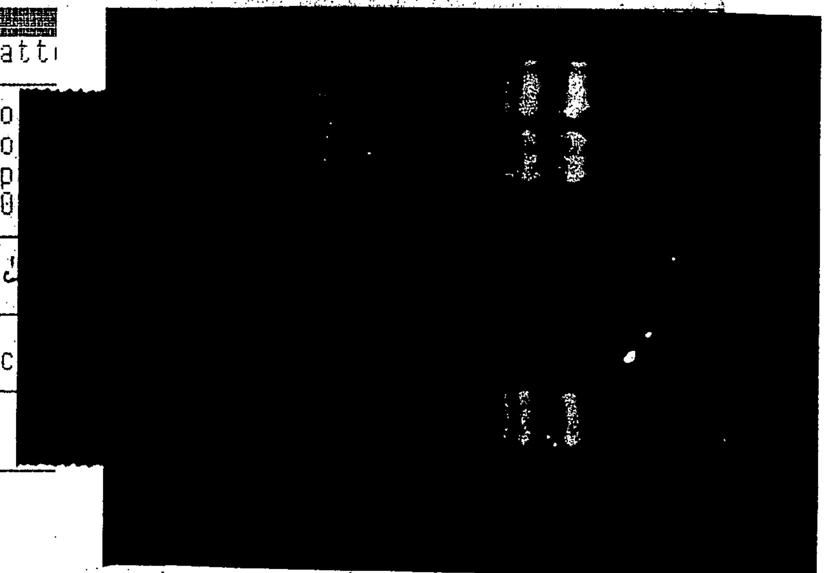
A:\WORK_001   Scan   Functions: Scan

wl	Abs	Pick
261.0	0.1627	pk
280	0.0865	

1.88

A = 3.254
= 130μg/ml.
= 130ng/μl.
10μl = 1.3μg.
= 4.5μg in 35μl





[redacted]

So process the rest of the RNA as a week tomorrow. But run 5μl on a gel with 5'

First injections for rabbits 26 + 27
↓ ↓
Perox (26) Gst 27 Hom

Use all of sol. left ... for 26, + 2 tubes of ... (#10 + 11) for #27. → 1½ ml + 1½ ml FCA.

Prepare more poly(A)$^+$ from total RNA.

for 1 ml

Make 2 x Binding Buffer. 20 mM TRIS-HCl pH 7.5 (1M) 20 μl
1 M LiCl (8M) 125 μl
2 mM EDTA (0.5M) 4 μl
H_2O 851 μl

for 2 ml 2 x Binding Buffer 40 μl TRIS (1M)
250 μl 8M LiCl
8 μl 500 mM EDTA
1702 μl H_2O (851 x 2)
2 ml

2 x 200 μl aliquots of total RNA processed → 10 μg each

Pool all the A+, scan + run on gel.

Also did ... prep on 200 μl tot. RNA → 10 μl. Run 1 lane.

Gel. 1) ...
2) –
3) Tot. 2 μl
4) 1st Pool A+ 2 μl → 80 V. Stain
5) 2nd prep. 1 μl
6) —
7) 1st ... 5 μl
8) 2nd .. .. 5 μl

cDNA synthesis from ~ 5μg poly(A)+ RNA.

Follow the strategene protocol & leave to precipitate for 1½ day @ -20°C (at lunch tomorrow).

12. 6. 96.

At Oslo 96.

13. 6. 96.

Pelleted cDNA 60 min, 4°C, 10,000g.
washed pellet 70% EtOH, dried & resuspended in 8 μl EcoR1 adaptors. Ligated O/N as per protocol 8°C.

14. 6. 96.

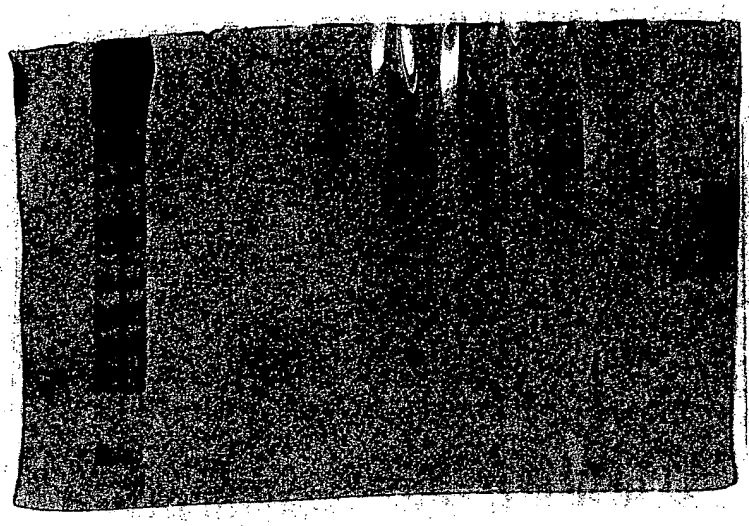
Kinase & Xho1 digestion of cDNA prior to spin column.

Make 6% acrylamide gel in 1x TBE for fraction analysis.

Stock acrylamide is 40%

for 10 ml	1 ml	10xTBE
	1.5 ml	Acrylamide/Bis.
	7.5 ml	H_2O
	10 ml.	

100 μl APS 10, 10 μl TEMED.



Ligated cDNA was passed thru Sephacryl S-500 column.
6 fractions taken = 60 µl.

5 µl of each → gel.

Gel run in 1× TBE, 150 V ≃ 45 min till BPB at bottom

Stained in BioRad after stain.

Gel.			
1)	—		
2)	λAt 5 µl.		
3)	—		
4)	Frac 1	very Hi.	
5)	2	800 bp → 3 kb	use 2+3.
6)	3	500 bp → 3 kb	
7)	4	"	
8)	5	200 bp → 3 kb.	
9)	6		
10)	—		

fractions extracted with P/C ×1.
C/IAA ×1 + EtOH ppt'd. −20°C
over the weekend.

Pellet the precipitating DNA 10,000 g, 4°C, 1 hr.
Resuspend the DNA pellet in 4 ml st. H_2O.
Quantify on Ethidium Bromide Plate.

Set up ligation O/N 12°C.

Prepare host strain for packaging reaction.

XL1B MRF' - plate out onto LB Tet Agar. (12.5 mg/ml)

Inoculate 50 ml LB Tet 10mM $MgSO_4$, 0.2% maltose.
↳ 37°C 4-6 hr OD 0.1.
↳ Pellet 500 g 10 min.
↳ 25 ml 10mM $MgSO_4$.

VCS 257 → LB
SOLR → LB kan (50 mg/ml)

Make 20% maltose in st. H_2O in 2 liter sterilin.
10mM $MgSO_4$ - autoclave.
st. H_2O.
Kanamycin stock (50 mg/ml).

20% Maltose – for 50ml 10g + Zero Stock.
make 25ml – 5g.

		for 50ml LB Tet Mgso4
LB let plates – 500ml –	5g NaCl	500mg NaCl
	5g Tryptone	500mg Trp.
	2.5g Yeast Extract	250mg YE
	10g Agar	125mg $MgSO_4.7H_2O$
	↳ pH 7.0	PH 7.0

$MgSO_4 = 246.5$. 10mm = 2.5g/l

LB top – 500ml	5g NaCl	25ml 2.5	
	5g Tryptone	2.5	10mM $MgSO_4$
	2.5g Yeast Extract	1.25	1.25g → 500ml
	3.5g Agarose	1.75	
	↳ pH 7.0		

NZY Agar plates – 500ml –
- 2.5g NaCl
- 1g $MgSO_4.7H_2O$
- 2.5g Yeast Extract
- 5g Casein Hydrolysate
- 7.5g Agar

NZY top – 500ml –
- 2.5g NaCl
- 1g $MgSO_4.7H_2O$
- 2.5g Yeast Extract
- 5g Casein Hydrolysate

Poured 3 dishes of NZYA 51TRL [redacted]
20 LB tet plates

SM buffer - 200ml.

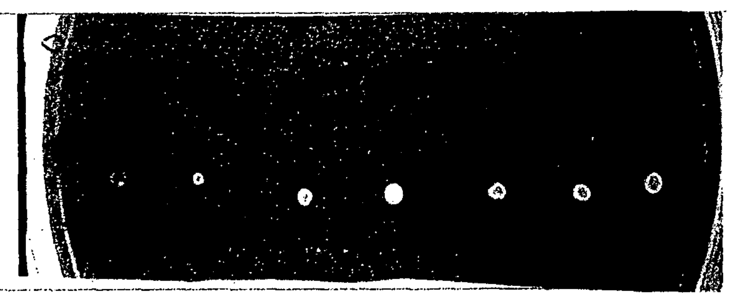
1.16g NaCl
400mg $MgSO_4.7H_2O$
10ml 1M TRIS (pH7.5) 1.21g TRIS
1 ml 2% Gelatin 20mg Gelatin.

Fractions 1, 2 & 3 were pooled into 5µl H_2O, since these all had no small cDNA.

0.5µl was spotted onto EtBr plate with DNA standards

Appears to be plenty of cDNA. Conc at 150ng/µl.

Set up ligation & store the rest of the cDNA. -80°C

[redacted]  [redacted]

c 100ng insert + 1µg vector.

Ligation 1
- 0.75µl cDNA
- 0.5µl 10x Buffer
- 0.5µl rATP
- 1µl Uni-ZAP
- 2.75µl H_2O
- 0.5µl Ligase.

Ligation 2 Dilute cDNA x3 use 1µl.
- 1µl cDNA.
- 0.5µl 10x
- 0.5µl ATP
- 1µl ZAP
- 1.5µl H_2O
- 0.5µl Ligase.

Grow plating cells - 50 ml LB Mg + 0.2% maltose + 12 μg/ml Tet.
→ add colony of XL1B MRF', → 37°C 200 rpm.

Lysates → 4°C.

Today start with plating cells + do packaging etc. tomorrow because of [illegible] [illegible].

Cells grown to OD 0.598.
Pellet 1500g in 2 × 25ml aliquots @ 4°C
Resuspend in 15ml 10mM $MgSO_4$ → OD [illegible].
Store on ice @ 4°C.

Dilute tomorrow to package.

Packaging of library. 3 μl of lysate 1 + 3 μl of lysate 2 were packaged with 1 tube each of Gigapack III for 90 min.
Remainder of lysate → -80°C.

Make 1ml 0.5M IPTG in water.
1ml X-Gal 250mg/ml in DMF.

IPTG = 238.3   1M = 238.3mg/ml
0.5M = 119mg/ml.

Plating cells OD = 1.4 v = 15ml.   i.e. → 42ml for OD 0.5
1ml → 2.8ml
0.33ml → 0.933ml
0.33ml + 0.6ml.

Plated packaged 1 & 2 libraries on NZY X-Gal / IPTG as in Stratagene manual, in 2.5ml NZY TOP (3ml better)

1µl Neat, 1/10, 1/100 & 1/1,000 for 1 & 2.

Dilutions made in SM. Stored at 4°C.

Plates → 37°C o/N.

Also transformed BL21-DE3 with plasmid from pET/GST 27 #1 - plasmid prep from 9/4/96. Use this to make new protein prep.

Unused plating cells → on ice @ 4°C room.

Tomorrow - amplify library & make plates for screen also.

GST pET transformations ok → 4°C for next week.

Library titres ok. still % of blues on each plates.

Lib #1 1/10 = 73 plaques 0 blues 730/µl for 500µl = (365,000 pfu) or 1,150,000
2 1/10 = 178 " 3 blues 890,000
1,050,000

Also have other library to be packaged.

≃ 2,700,000 pfu.
≃ 1ml @ 1.1ng.

for immersion – grow 37°C 3-4 h.

N. [illegible] in D.m 10ml – dry on paper.

Overy. ≥ 4 h 37°C.

Rinse lid 20 min.

Cool, mark + [illegible].

western blot.

Peel → 4°C.

[redacted]

Plate library on 2 dishes for amplification.

Re-count of 1/100 plates for library titre.
Blue background is small ≈ 2%

1 1/100 = 33, 0 blues 3,300 pfu/µl 1,650,000 pfu.
2 1/100 = 22, 2 blues 2,200 pfu/µl 1,100,000 pfu.
i.e. 2,750,000 pfu in 1ml.

Use 3ml top agar for 90mm plate 64cm²
Bioassay dish = 591cm² ≡ 27.7 ml top agarose.
use 30ml, i.e. 10x vols. of plate

30ml top agarose.
2ml plating cells @ OD 0.5.

By observation - 2 µl of lib / 90 mm plate is OK ≡ 20 µl for dish.
20 µl = 66,000 pfu 1 on plate more densely i.e. 170,000
= 44,000 pfu 2.

plating cells - 330 µl + 660 µl $MgSO_4$
for 4ml 1.32ml 2.64 µl

= 51 µl lib 1
= 77 µl lib 2

#1	#2
2 ml cells	2 ml cells
51 µl 1/2 lib 1	77 µl 1/2 lib 2
30 ml TOP Agarose	30 ml TOP Agarose

Grow o/v 37°C, overlay with 30 ml SM, rock o/N 4°C.
Remove SM, wash plate 7 ml SM pool. $CHCl_3$ to 5% v/v, mix RT 15 min.
Pellet 10 min 500g. SN → $CHCl_3$ 0.3% v/v 4°C.
Store -80°C 7% DMSO.

Make 2 x 200 ml LB for overnight ferm.

for 400 ml LB 4g NaCl
4g Tryp
2g Yeast Ext → pH 7.0

Make Ampicillin @ 100 mg/ml.
Carbenicillin @ 100 mg/ml

Set up 2 x 5 ml o/n cultures from plate. 100 µg/L carbenicillin

Wash the ampicillin colony plates in 30 ml SM with shaking @ 4°C for 6 h. Then add $CHCl_3$ to 5%. → RT 15 min.

Pellet 10 min, 500g.
SN → Falcon tube + 0.3% $CHCl_3$.

Also inoculate 2x200 ml LB CARB 100 1 flask 200 ml o/N.
1 flask colony.

Grow 2-3 h x o/N 180 rpm to 1mM.

Lib 1 → 32 ml. (+1.6 ml $CHCl_3$) → 29 ml 87 µl $CHCl_3$
Lib 2 → 29 ml. (+1.45 ml $CHCl_3$) → 27 ml 81 µl $CHCl_3$

PCR on 5 plaques from plate 1, 5 from plate 2.

T3 + T7 primers are @ 15pm/μl.

Pick plaque → 100μl SM – remove 5μl for template.

PCR mix	9.0 μl	25mm $MgCl_2$
	5 μl	10x Buffer
	8 μl	1.25mm dNTPs.
	3 μl	T3
	3 μl	T7
	5 μl	Template
	0.5 μl	TAQ.

– use MS mix. '11x'

		½ vol.
4.5 μl	MS mix.	2.25
5 μl	Temp.	2.5
0.5 μl	TAQ	0.25
3 μl	T3	1.5
3 μl	T7	1.5
34 μl	H_2O	17
50 μl.		25 μl

Result – run on gel – all have 800bp band + additional fainter bands 500bp → 2kb.
PCR components contaminated?
Do another PCR mainly with different primers to check.

Master mix for 11 tubes = 24.75 μl MS 11x
2.75 μl Taq.
16.5 μl T3
16.5 μl T7
187 μl H_2O
247.5 μl.

22.5 μl Mix + 2.5 μl Temp.

PCR – 25 x 94 – 1, 50 – 1, 72 – 2

Plating cells grown o/N in 50 ml LB Mg, Tet. 30°C.

Pelleted → 10 ml 10mM $MgSO_4$ → OD 2.1

Take 1ml + 3 ml 10mM $MgSO_4$ → OD 0.5 for plating for immunoscreen.

(OD 0.5)
Screen 1 big dish — 2ml plating cells, 40 μl Lib 2, 25 μl Lib 1 } → [redacted]
30ml TOP Agarose.

on @ 10-15am.

Grow 3-4 h, overlay with filter in 10mM IPTG.
Grow 3-4 h & remove filter.

IPTG = 238.
10mM = 2.38 mg/ml.
for 100ml - 238 mg, in st. H_2O.

After 3½ h plaques just visible. Overlay with N/C + IPTG for 4 h. Lift & (store in TBS o/N.) Detect tomorrow.

D.D. ran some [illegible] protein from [illegible]'s [illegible]. Looks OK.

Filter was marked in each corner. Lifted & rinsed in 1xTBS → Blocked up in 200 ml 3% Marvel, 1xTBS.

Inc o/N 4°C 21F 1/5,000 for 200 ml = 40 μl Ab.

[redacted]

26

[redacted]

Wash filter as usual. → 2'y Ab 1/5,000 1h.
Wash as usual.
Wash 100mM TRIS 9.5 + develop.
→ 15' +ve.

Run gel & blot for new Abs.

1) SDS FB.
2) 24P 20l
3) 14H 20l. Run c 150V
4) 23H 20l. Blot.
5) Overexpressed 5l. Store blot -20°C until Abs ready.
6) SDS FB.
7) 24P Results – 27 – curiously picks out band ≈ 3 kt. GMs.
8) 14H No reaction with overexpressed protein
9) 23H 26 – nothing.
10) Overexpressed. ∴ Boost & check again

Abs. arrived, so block c inc c Ab. $\frac{1}{1,000}$ o/N.
Record ≈ 8ml of each. → -20°C 1ml aliquots.

Picked 17 1'y plaques → 1ml SM + 20l $CHCl_3$.
Vortex & store 4°C. Assume titre ≈ 6×10^7 pfu/ml.
i.e. 6×10^4 pfu/μl.

Develop western blots. Wash as usual. 2° Ab 1/10,000, 1 hr.

Secondary screen — assume 1° stocks are c 6×10^4 pfu/μl

i.e. 10 μl = 6×10^5 pfu.

Need 6×10^1 – 6×10^2 pfu for 1 plate.

i.e. 10 μl of 10^{-3} – 10^{-4} stocks to OK for 2° screen. 10^{-1} is 1 μl per ml.
+ dilute x 10 for 10^{-4}.

200 μl cells, 10 μl phage, 3 ml TOP Agarose.

↳ 37°C 3½ – 4 hr.

Overlay with N/C + IPTG + inc. 37°C o/N.

10^{-4} plates OK. Lift N/C, wash TBS, block 3% M.P/TBS inc. 2° F 1/5000 1 hr. for 400 ml = 80 μl.

		-3	-4		-3	-4
Pairs -	1	✓	–	9		✓
	2	✓	–	10		✓
	3	✓	–	11		✓
Pick 10^{-3} plates	4		✓	12	✓	–
for 10^{-4} – ves	5		✓	13	✓	✓
	6	✓	–	14	✓	–
	7	–	–	15	✓	–
	8		✓	16	✓	–

Pick 2'y screen.

where possible pick singles for rescreen & expansion.

If too dense, then pick all patches from 1 plate, pool & re-screen next week.

Also Hex-GH colonies from frozen glycerol pellets of overnight CST II transformation.

Use pellets by counting ~ 10 ml broth. Pellet debris & do CDNB assay.

Clear = 0.051

SN > 0.105.

Apply to Hex-GH as usual. Assay all fractions for CDNB activity.

Did not bind! So for the moment discard this & use frozen stuff for Ab.

Procedure for Single-clone excision.

1) Streak XL1B MRF' on LB tet. (12 µg/ml)
SOLR LB kan (50 µg/ml)

2) Grow o/N colony of XL1BMRF' tet Mal MgSO4.
SOLR + LB kan.

Make 100 ml LB + 500 ml flask.

Need Ampicillin Plates

5 ml LBs

o/N liquid cultures of XL1B MRF' maltose/Mg/Tet
+ SOLR kan

Pellet 500g 15 min + supernatant + 10mm MgSO4
to OD of 1.0.

Ex-assist is @ 10^7 pfu/ml. — use 1 µl.
Assist phage picks are @ 10^4 pfu/ml. — use 100 µl + 150 µl SM.
200 µl XL1BMRF' @ OD 1

↳ 37°C, 15 min + filter free

Add 3 ml LB + inc. 2½ h 37°C with shaking.

↳ 70°C, 20 min — 1000g 15 min. Keep SN.

for OD1

SOLR (25ml MgSO₄) OD = 2.02 → 50ml.
XL1B (25ml MgSO₄) OD = 2.5. → 62.5ml.

Performed plaque assay on the 12 single plaque picks isolated last Friday. As per protocol, took and 10 μl & 1 μl of excised phagemids with 200 μl SOLR cells & plated different amounts of this onto LB Amp plates. → 37°C o/N.

Plenty of colonies on all the plates. Grow these up o/N in LB AMP KAN. for plasmid preps tomorrow.

Today – re-screen the 1° picks I could not purify by 2° screen, i.e. # 7, 3, 12.

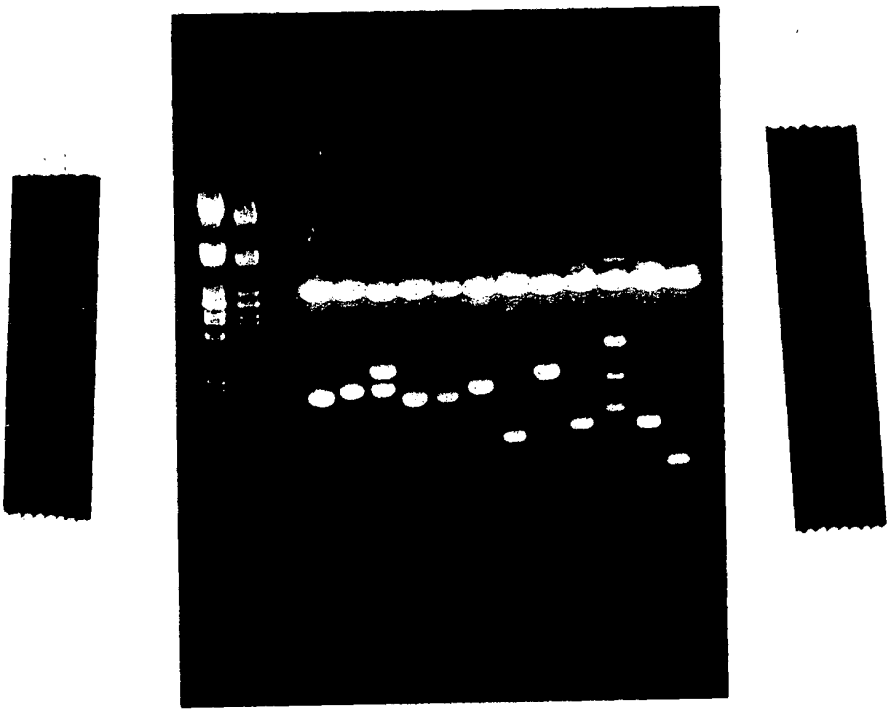
Dilute to 10^{-3} & 10^{-4} & use 10 μl phage with 100 μl cells OD 1 & 3 ml top agarose.

Incubate 4 hrs, overlay with N/C disc + IPTG & inc O/N.

Also – gel purify 24 kD band on 2 0.75 mm 15% Tris/Tricine gels.

Load 100 μl of 9/2 # 10 on each. Fix 15 min. Stain 10 min – excise band & inc. in Porage buffer o/N.

→ Bands not clearly resolved – must be overloaded. So no use for these gels.



New Wiz. preps. from 12 O/N 50ml cultures.

3ml culture → 50µl DNA.

Digest 5µl → 30µl EcoR1 / Xho1.

5µl DNA		39µl 10x
3µl 10x	for 13 master mix	13µl EcoR1
1µl EcoR1		13µl Xho1
1µl Xho1		260µl H2O
20µl H2O		
30µl		x mix 25µl for each digest.

Run a 1% Agarose / TBE gel.

Digest λ/Pst1 marker. λ is 0.5µg/µl. for 50ng - 100µl.

100µl λ (50ng)
20µl 10x Buff.
10µl Pst1
70µl H2O
200µl
↳ 37°C 1 h.

Clones are 1, 2, 4, 5, 6, 8
9, 10, 11, 13, 14, 16.

Rerun #3-12 O/N.
No +ve for #7.

Wiz. DNA → −20°C for later seqing.

Check plasmids 1 & 2 prior to sequencing.

1 µl → 10 µl. & run 1, 2, 4 µl on gel.
2 & 6 µl λ Pst + 100 ng/µl.

200 ng λ Pst → 12.5 ng 3 kb.
600 ng λ Pst → 37 ng 3 kb.

Sequencing DNA 5 µl of 100 ng/µl per reaction.

1 µl $\frac{1}{10}$ ≃ 37 ng. i.e. 370 ng/µl of stock

Take 1.3 µl of stock → 5 µl. Do F & R on 1 & 2
& do further restriction mapping & expression studies.

1.3 µl DNA + 3.7 µl H_2O.

Rapid mini for plasmids 1 & 2 (A & B for both).

1 µl of excised phagemids + 200 µl SOC & cells → 37°C 15 min.
Title on dried plates (Amp 50/50) & grow O/N.

Make 200 ml LB Agar.		100 ml LB (20 × 5 ml)	
2 g	NaCl	1 g NaCl	1 µl 5N
2 g	Typtone	1 g Typ	split
1 g	YE	0.5 g YE	
2 g	Agar.		

↪ pH 7.0 & autoclave.

Ran #9 8.2.96 on 2 x 15% S<VJ gels.

176 ul + 176 ul buffer split between the 2.

Fix 10 min, 45% methanol, 10% HAc
Stain B+Bl 30 min

Excised bands → 500 ul Porzyn buffer — homogenised →
37°C O/N.

Clones 1, 2, 4, 5, 6, 8, 9, 10, 11, 13, 14 & 16 induced.

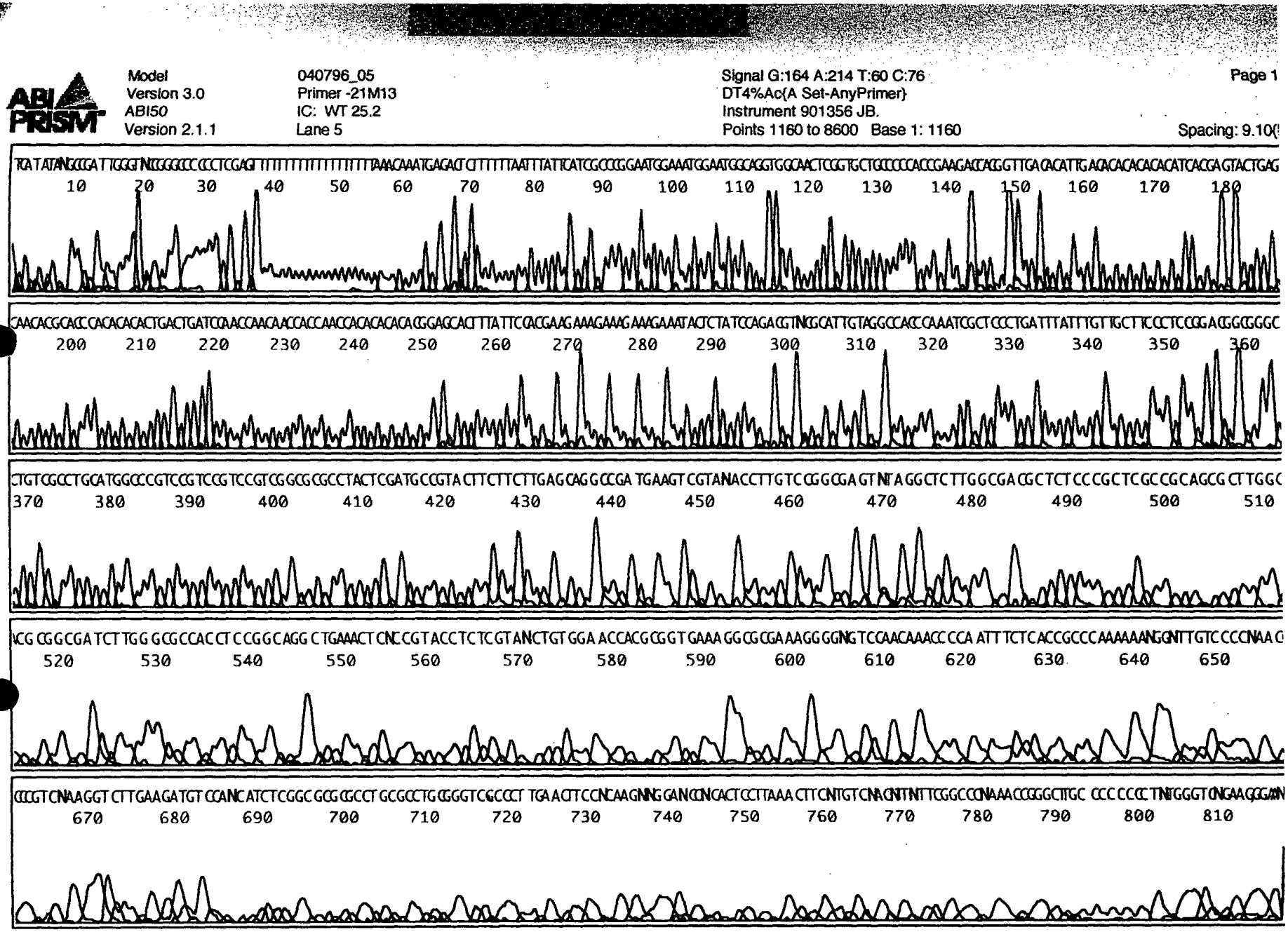
Inoc. 5ml LB Amp/Kan with 100 ul of frozen cultures.
Grow to OD 0.6 → 1 & add IPTG to 1mM. Grow
3 h.

– In fact – grown for 2½ h, still OD ≈ 0.2. So add IPTG
+ grow for an additional 6 h. Pellet & freeze until Monday.

Excised protein → 40 ul. Add 20 ul 4tube → -80°C.

Prepare glycerols of GST clones from preps. used for WT
DNA preps. Also keep 1 ml of culture & pellet for
control c.f. induced cultures.

700 ul culture + 300 ul 50% glycerol.
350 + 150.


Model
Version 3.0
ABI50
Version 2.1.1
040796_05
Primer -21M13
IC: WT 25.2
Lane 5
Signal G:164 A:214 T:60 C:76
DT4%Ac{A Set-AnyPrimer}
Instrument 901356 JB.
Points 1160 to 8600 Base 1: 1160
Page 1
Spacing: 9.10{
10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180
200 210 220 230 240 250 260 270 280 290 300 310 320 330 340 350 360
370 380 390 400 410 420 430 440 450 460 470 480 490 500 510
520 530 540 550 560 570 580 590 600 610 620 630 640 650
670 680 690 700 710 720 730 740 750 760 770 780 790 800 810

ABI PRISM

Model
Version 3.0
ABI50
Version 2.1.1

040796_09
Primer reverse
IC: GTT 25.2
Lane 9

25.2

Signal G:158 A:196 T:43 C:69
DT4%Ac{A Set-AnyPrimer}
Instrument 901356 JB.
Points 1160 to 8000 Base 1: 1160

Pag

Spacing: 9.2